Covalent Coupling of the Variable Loop of the Elongator Methionine tRNA to a Specific Lysine Residue in *Escherichia coli* Methionyl-tRNA Synthetase†

Oscar Leon‡ and LaDonne H. Schulman*

Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Received September 10, 1986; Revised Manuscript Received December 8, 1986

ABSTRACT: A lysine-reactive cross-linker has been coupled to the minor base 3-(3-amino-3-carboxypropyl)uridine in the variable loop of the *Escherichia coli* elongator methionine tRNA ($tRNA_m^{Met}$). Incubation of the derivatized tRNA with *E. coli* methionyl-tRNA synthetase (MetRS) resulted in covalent coupling of the protein and nucleic acid and loss of amino acid acceptor activity of the enzyme. One mole of tRNA was cross-linked per mole of enzyme inactivated. Enzyme activity was largely restored by release of the bound tRNA following cleavage of the disulfide bond in the cross-linker with a sulfhydryl reagent. The cross-linking reaction was effectively inhibited by unmodified $tRNA_m^{Met}$ but not by noncognate $tRNA^{Phe}$. The covalent complex was digested with trypsin, and the resulting tRNA-bound peptides were isolated by anion-exchange chromatography. The cross-linked peptides were released from the tRNA by cleavage in the disulfide bond of the cross-linker and purified by reverse-phase high-pressure liquid chromatography, yielding one major peptide plus several minor peptides. Amino acid analysis indicated that the major product was an octadecapeptide cross-linked to $tRNA_m^{Met}$ through lysine residue 596 in the primary sequence of MetRS. The N-terminal sequence of the peptide was determined to be Val-Ala-Leu-Ile-Glu-Asn-Ala-Glu-Phe-Val, corresponding to residues 582–591 in MetRS. The procedures described here should be applicable to the determination of peptide sequences near the variable loop of other tRNAs containing the 3-(3-amino-3-carboxypropyl)uracil base when such tRNAs are bound to specific proteins.

Covalent cross-linking of proteins and nucleic acids provides an approach to the identification of the structural regions that are in close proximity when two macromolecules are bound in a specific complex in solution. Several techniques have been applied to affinity label proteins that interact with transfer RNAs, and in a few cases, the sequences of cross-linked peptides have been obtained. Yeast phenylalanyl-tRNA synthetase, *Escherichia coli* methionyl-tRNA synthetase (MetRS),[1] and *E. coli* tyrosyl-tRNA synthetase have been cross-linked to the periodate-oxidized 3′ termini of their respective cognate tRNAs, and peptide sequences near the active sites of these enzymes have been identified (Renaud et al., 1982; Hountondji et al., 1985, 1986). 3′-Oxidized tRNA has also been used to affinity label a kirromycin complex of elongation factor Tu (Van Noort et al., 1984), and a specific histidine residue at the aminoacyl-tRNA binding site of this protein has been coupled to N^{ϵ}-(bromoacetyl)lysyl-tRNA (Duffy et al., 1981).

Many tRNAs contain naturally occurring modified bases that can be used to attach cross-linking reagents to specific internal sites in the tRNA structure. Photoaffinity reagents have been coupled to 4-thiouridine residues (Budker et al., 1974; Gorshkova et al., 1976; Wetzel & Soll, 1977) and to 3-(3-amino-3-carboxypropyl)uridine (acp^3U) (Kao et al., 1983). Covalent reaction of such tRNA derivatives with aminoacyl-tRNA synthetases and elongation factor Tu has been observed; to date, however, no peptide sequences have resulted from these studies.

We have previously coupled a cleavable lysine-reactive cross-linker to modified single-stranded cytidine residues in *E. coli* $tRNA^{fMet}$ and obtained high-yield cross-linking to the tRNA binding site of *E. coli* MetRS (Valenzuela et al., 1984; Schulman et al., 1981a,b). The sequences of four of the MetRS-cross-linked peptides have recently been reported (Valenzuela & Schulman, 1986). This was the first successful identification of peptides located near internal regions of a tRNA bound to a specific protein. In this paper, we describe procedures for coupling the same cross-linker to the acp^3U residue at position 47 in the variable loop of *E. coli* $tRNA_m^{Met}$ and report the sequence of the major MetRS peptide that is cross-linked to this affinity labeling tRNA derivative.

Materials and Methods

Materials

Dithiobis(succinimidyl propionate) (DTSP) and Sequenal-grade trifluoroacetic acid were obtained from Pierce Chemical Co. *N,N′*-Dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and spectrophotometric-grade dimethyl sulfoxide were purchased from Aldrich Chemical Co. HPLC-grade acetonitrile was from Fisher, and water was purified in a

† This work was supported by Research Grant GM16995 from the National Institutes of Health. Partial salary support for L.H.S. was provided by National Cancer Institute Grant P30CA13330.

‡ Present address: Department of Molecular Biology, Faculty of Science, University of Concepción, Casilla 2407, Concepción, Chile.

[1] Abbreviations: $tRNA_m^{Met}$, *E. coli* elongator methionine tRNA; $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; DTSP, dithiobis(succinimidyl propionate); DTSP-$tRNA_m^{Met}$, $tRNA_m^{Met}$ modified by coupling DTSP to the 3-(3-amino-3-carboxypropyl)uridine residue at position 47 from the 5′ terminus; acp^3U, 3-(3-amino-3-carboxypropyl)uridine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; GdmCl, guanidinium chloride; DTT, dithiothreitol; TPCK, N^{α}-tosylphenylalanine chloromethyl ketone; DEAE, *N,N*-diethylaminoethyl; ODS, octadecylsilane; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-1933$01.50/0

MilliQ reagent-grade water system from Millipore Corp. DTT and β-propiolactone were obtained from Sigma Chemical Co., and sublimed sulfur was from Mallinckrodt. [^{35}S]DTSP was synthesized by the method of Lomant and Fairbanks (1976). ^{35}S-Labeled elemental sulfur and [^{35}S]methionine were obtained from Amersham. [^{14}C]Methylamine was from New England Nuclear. Superfine Sephacryl S-200 and SP-Sephadex C-50 were purchased from Pharmacia Fine Chemicals and collodion bags (M_r 25 000 cut-off) from Schleicher & Schuell. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K12 strain EM20031 as described before (Schulman & Pelka, 1977a). TPCK-treated trypsin was obtained from Worthington Biochemical Corp. and further purified on SP-Sephadex C-50 by the procedure of Beeley and Neurath (1968). Deacylated *E. coli* $tRNA_m^{Met}$ having a specific amino acid acceptor activity of 1650 pmol/A_{260} unit and $tRNA^{Phe}$ (1750 pmol/A_{260}) were purchased from Subriden.

Methods

Preparation of DTSP-Modified $tRNA_m^{Met}$. Unlabeled DTSP or [^{35}S]DTSP (35–450 cpm/pmol) was dissolved in fresh Me_2SO at a concentration of 2.6 mg/mL immediately before use. *E. coli* tRN_m^{Met} was dissolved in 0.2 M Hepes buffer, pH 7.8, at a concentration of 25 A_{260} units/mL. The tRNA and DTSP solutions were mixed in a 1.0 to 0.75 ratio and incubated at room temperature for 15 min in a siliconized polypropylene centrifuge tube. The reaction was stopped by addition of 2 volumes of ethanol, and the sample was chilled for 10 min at −20 °C and centrifuged. The modified tRNA was redissolved in 0.1 M sodium acetate, pH 6.0, at a concentration of 20 A_{260} units/mL, transferred to a fresh siliconized centrifuge tube, and precipitated as before. The tRNA was reprecipitated 3 more times from 0.1 M sodium acetate, pH 6.0, and dissolved in 10 mM $MgCl_2$ just before use.

The amount of reactive ester remaining attached to the tRNA following the coupling reaction was determined by quenching samples of unlabeled DTSP-$tRNA_m^{Met}$ with [^{14}C]-methylamine and measuring the incorporation of ^{14}C into a TCA-insoluble form. The modified tRNA was incubated in 0.2 M Hepes, pH 7.8, and 5 mM [^{14}C]methylamine at 25 °C for 1 h in the dark. Aliquots were removed, added to 1 mL of 10% TCA, mixed, filtered through nitrocellulose disks, and washed with 15 × 5 mL portions of 5% TCA, followed by 5 mL of ethanol. The filters were dried and counted in Econofluor (New England Nuclear). Blanks were determined from samples of unmodified $tRNA_m^{Met}$ treated in the same way.

Hydrolysis of the reactive *N*-hydroxysuccinimide ester group of DTSP-$tRNA_m^{Met}$ was carried out by incubation of the modified tRNA in 0.1 M Hepes, pH 9.0, at 37 °C for 4 h followed by ethanol precipitation. The tRNA was reprecipitated from 0.1 M NaCl and stored frozen in 10 mM $MgCl_2$.

Kinetic parameters for aminoacylation of $tRNA_m^{Met}$ derivatives were determined as described by Schulman and Pelka (1983) with tRNA concentrations in the range 0.25–3 μM. Initial rates of aminoacylation were linear with time and proportional to the MetRS concentration in the range used.

Site of Attachment of DTSP to $tRNA_m^{Met}$. [^{35}S]DTSP-$tRNA_m^{Met}$ was converted to the corresponding sulfhydryl derivative by incubation with 20 mM DTT in 0.1 M Tris, pH 7.5, at 37 °C for 90 min. Carrier unlabeled tRNA was added to a final concentration of 20 A_{260}/mL, and the mixture was precipitated by addition of 2 volumes of ethanol. The tRNA pellet was taken up in 0.1 M sodium acetate, pH 6.5 (13 A_{260}/mL), and digested with T_1 RNase (500 units/mL) at 37 °C for 90 min. Calf intestinal alkaline phosphatase was added (4 units/mL) and the incubation at 37 °C continued for another hour. The digest was diluted 10-fold with 7 M urea, 20 mM sodium acetate, pH 6.5, and 1 mM DTT and injected onto a 7.5 × 75 mm column of Bio-Gel TSK-DEAE 5PW equilibrated with the same buffer. The oligonucleotides were eluted with a linear salt gradient from 0 to 0.3 M NaCl over 3 h at a flow rate of 0.35 mL/min. Two-minute fractions were collected and then diluted with 5 mL of ACSII scintillation fluid (Amersham), and the ^{35}S radioactivity was measured. The absorbance at 260 nm was monitored with a Gilson Holochrome detector. The site of attachment of [^{35}S]DTSP to $tRNA_m^{Met}$ was determined by comparison of the elution position of the ^{35}S-labeled oligonucleotide with the elution positions of the A_{260} T_1 RNase oligonucleotide markers of known sequence.

Cross-Linking of DTSP-$tRNA_m^{Met}$ to MetRS. Reaction mixtures contained 1–4 μM MetRS and 3–15 μM DTSP-$tRNA_m^{Met}$ in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$. Solutions were incubated in siliconized culture tubes at 25 °C. Aliquots were removed at various times, quenched by incubation with 50 mM glycine for 30 min at 25 °C, and assayed for residual enzyme activity and/or binding of ^{35}S-labeled DTSP-$tRNA_m^{Met}$ to nitrocellulose filters (Millipore, type HA) in the presence of high salt as described before (Schulman et al., 1981a). The cross-linked tRNA was released from the covalent complex by cleavage of the disulfide bond of the cross-linker on addition of DTT to 25 mM and further incubation of the reaction mixture at 37 °C for 90 min.

Control experiments were carried out with enzyme incubated in the absence of DTSP-$tRNA_m^{Met}$ and in the presence of an equivalent amount of unmodified tRNA. Competition experiments were carried out at 25 °C with 1.1 μM MetRS in 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 6.6 μM DTSP-$tRNA_m^{Met}$, and 40 μM unmodified $tRNA_m^{Met}$ or $tRNA^{Phe}$. The unmodified tRNAs were added to the enzyme solution immediately prior to addition of DTSP-$tRNA_m^{Met}$.

Isolation of Peptides Cross-Linked to DTSP-$tRNA_m^{Met}$. Cross-linking was carried out in 3.2 mL of 12 mM Hepes, pH 8, and 10 mM $MgCl_2$ containing 4.2 μM MetRS and 14 μM ^{35}S-labeled DTSP-$tRNA_m^{Met}$ (35 cpm/pmol). The solution was incubated at 25 °C for 30 min. Glycine, pH 7, was added to a final concentration of 50 mM and the incubation at 25 °C continued for another 30 min. Aliquots were withdrawn for measurement of residual enzyme activity and determination of the amount of cross-linked complex by nitrocellulose filtration. The remaining reaction mixture was concentrated in a collodion bag to 2.6 mg/mL protein and dialyzed against 0.1 M ammonium acetate, pH 6.5, and 0.1 mM $CaCl_2$ for 2 h. The protein was digested with 3% RNase-free trypsin (w/w) for 7 h at 25 °C. The tryptic digest was adjusted to 0.1 M NaCl by addition of 4 M NaCl, 3 volumes of ethanol were added, the solution was chilled at −20 °C for 10 min, and the tRNA was pelleted by centrifugation for 10 min. The pellet was redissolved in 7 M urea, 0.1 M NaCl, and 0.05 M ammonium acetate, pH 6.5 (buffer A), and injected onto a 75 × 7.5 mM Bio-Gel TSK-DEAE 5PW ion-exchange HPLC column equilibrated with the same buffer. The column was washed with a gradient from 0 to 20% B (B = 7 M urea, 1 M NaCl, 0.05 M ammonium acetate, pH 6.5) over 5 min followed by a 25 min wash with 20% B. This was followed by a second gradient from 20 to 100% B over 30 min. One-minute fractions were collected at a flow rate of 0.5 mL/min, and aliquots were taken for measurement of radioactivity. The radioactive fractions from the second gradient were pooled (7.5 mL) and dialyzed twice for 3 h vs. 2 L of 0.1 M sodium acetate, pH 6.5, and once vs. 2 L of 25 mM sodium acetate,

pH 6.5, overnight. The dialyzate was concentrated to 20 A_{260} units/mL and 0.3 M sodium acetate. Two volumes of ethanol were added, the solution was chilled for 20 min at −20 °C, and the tRNA was pelleted by centrifugation for 10 min. The pellet was reprecipitated once from 0.1 M sodium acetate, pH 6.5, and then dissolved in 1.5 mL of 0.1 M sodium acetate, pH 6.5, and 20 mM DTT and incubated at 37 °C for 90 min. The tRNA was precipitated by addition of 2 volumes of ethanol as before. The supernatant obtained following centrifugation (4.5 mL) was evaporated to 0.2 mL. One milliliter of water was added and the solution evaporated to 50 μL. GdmCl at 6 M (1.45 mL) was added and mixed thoroughly, and the solution was centrifuged in an Eppendorf tube for 5 min to remove small particles. The sample was injected onto a 250 × 4.6 mm Altex PTH-amino acid ODS reverse-phase HPLC column equipped with a 45 × 4.6 mm precolumn (Rainin) and preequilibrated with 0.1% TFA in water (v/v) (solvent A). The peptides were eluted at a flow rate of 0.65 mL/min on a Rainin gradient HPLC system with the following gradient profile: solvent A from 0 to 10 min followed by a linear gradient from 0 to 42% solvent B in 120 min [solvent B = 0.1% TFA in CH_3CN (v/v)]. A second linear gradient from 42 to 70% solvent B was run from 130 to 150 min, followed by a 10-min wash with 70% solvent B. The absorbance of the effluent at 210 and 280 nm was monitored on a Gilson Model 116 detector. One-minute fractions were collected, and aliquots (15 μL) were taken for measurement of radioactivity.

Amino Acid Composition Analysis and Peptide Sequencing. Amino acid analysis using the phenyl isothiocyanate precolumn derivatization procedure was carried out in a PICO-TAG work station (Waters Associates) at the Protein Sequencing Facility at Yale University as described by Stone and Williams (1986). Norleucine was added to each sample prior to hydrolysis to serve as an internal standard.

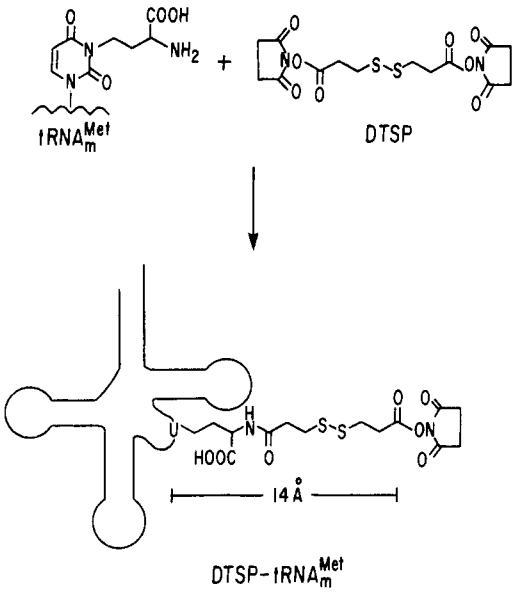
Peptide sequencing was carried out by automated N-terminal degradation with PITC on an Applied Biosystems gas-phase sequenator at Yale University (Stone & Williams, 1986). Amino acids were determined by the high-sensitivity PTH-amino acid detection method of Merrill et al. (1984).

RESULTS

Attachment of a Lysine-Reactive Cross-Linker to E. coli $tRNA^{Met}_m$. *E. coli* $tRNA^{Met}_m$ contains the minor base 3-(3-amino-3-carboxypropyl)uridine (acp^3U) at position 47 in the variable loop of the tRNA (Friedman et al., 1974; Ohashi et al., 1974). This minor base contains a chemically reactive primary amino group that can be readily coupled to *N*-hydroxysuccinimide esters (Friedman, 1973). We have prepared a lysine-reactive derivative of $tRNA^{Met}_m$ by coupling dithiobis(succinimidyl propionate) (DTSP) to the acp^3U base (Scheme I). The resulting amide bond is stable to base hydrolysis; however, the ester moiety of the cross-linker is readily hydrolyzed to the corresponding carboxylic acid derivative.

Incubation of $tRNA^{Met}_m$ with excess [^{35}S]DTSP in 0.11 M Hepes, pH 7.8, and 43% Me_2SO at 25 °C for 15 min led to attachment of 1 mol of cross-linker/mol of tRNA. Approximately 80–85% of the lysine-reactive *N*-hydroxysuccinimide ester group of the modified tRNA remained intact following the coupling reaction. The acp^3U base occurs in a sequence that gives rise to the oligonucleotide $m^7Gacp^3UCACAG$ on digestion of $tRNA^{Met}_m$ with T_1 RNase. Cleavage of the disulfide bond of [^{35}S]DTSP-$tRNA^{Met}_m$ with DTT followed by digestion of the sulfhydryl derivative with T_1 RNase and anion-exchange chromatography of the resulting oligonucleotides yielded the expected ^{35}S-labeled heptanucleotide (data not shown).

Scheme I: Attachment of DTSP to a Minor Base in $tRNA^{Met}_m$[a]



[a] The distance from the uridine ring of acp^3U_{47} to the carbonyl group of the *N*-hydroxysuccinimide ester is approximately 14 Å in the maximally extended form of the cross-linker.

Aminoacylation of Modified $tRNA^{Met}_m$. The reactive ester group of DTSP-$tRNA^{Met}_m$ was completely hydrolyzed by incubation of the modified tRNA in 0.1 M Hepes, pH 9, at 37 °C for 4 h. The kinetic parameters for aminoacylation of the ester-hydrolyzed derivative of DTSP-$tRNA^{Met}_m$ were determined and compared with the K_m and V_{max} values obtained with a control sample of $tRNA^{Met}_m$ subjected to the same modification procedure in the absence of DTSP and a sample of untreated $tRNA^{Met}_m$. The K_m value for the ester-hydrolyzed derivative (0.7 μM) was slightly lower than that observed for untreated $tRNA^{Met}_m$ (0.9 μM) and the Me_2SO-treated control (1.0 μM). V_{max} values were 1.6–1.7-fold higher for the control tRNAs than for the ester-hydrolyzed derivative of DTSP-$tRNA^{Met}_m$. Such small changes reflect only minor alterations in the interaction of the derivatized tRNA with MetRS. Larger differences in K_m and V_{max} (2–4-fold) are found for aminoacylation of the two naturally occurring isomers of *E. coli* $tRNA^{fMet}$, which differ by a single base in the variable loop at position 46 ($m^7G_{46} \leftrightarrow A_{46}$) (Daniel & Cohn, 1976; Schulman & Pelka, 1977b).

Cross-Linking of DTSP-$tRNA^{Met}_m$ to MetRS. Incubation of DTSP-$tRNA^{Met}_m$ with MetRS in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$ at 25 °C resulted in rapid formation of a covalent complex of protein and nucleic acid. Reactions carried out in the presence of excess tRNA were complete within 20 min, as measured by the retention of [^{35}S]DTSP-$tRNA^{Met}_m$ on nitrocellulose filters. The effective concentration of the lysine-reactive species decreases during the incubation due to hydrolysis of the *N*-hydroxysuccinimide ester group of the cross-linker. Approximately 25% of the input tRNA is cross-linked on incubation of MetRS with a 3-fold excess of DTSP-$tRNA^{Met}_m$ at pH 8 for 20 min.

Covalent complexes could be readily separated from excess unreacted tRNA by gel filtration on Sephacryl S-200 following quenching of the reaction with 50 mM glycine. Figure 1 shows the elution profile of the ^{35}S-labeled tRNA derivative following cross-linking to MetRS. Peak I contains the covalent protein–tRNA complex. Peaks II and III are derived from the free tRNA and appear in the profile of glycine-quenched

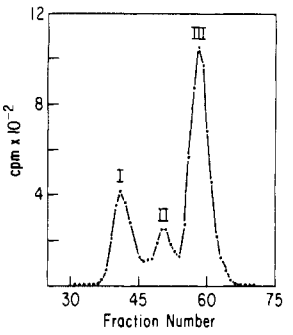
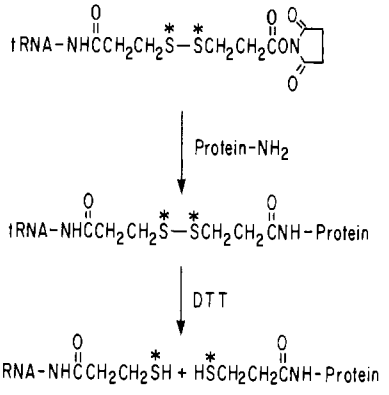


FIGURE 1: Isolation of the cross-linked complex of MetRS and DTSP-tRNA$_m^{Met}$ on Sephacryl S-200. MetRS (1 μM) and [^{35}S]-DTSP-tRNA$_m^{Met}$ (3 μM) were incubated in 20 mM Hepes, pH 8.0, and 10 mM $MgCl_2$ at 25 °C for 30 min. Glycine was added to a final concentration of 50 mM and the incubation at 25 °C continued for 30 min. The reaction mixture (0.5 mL) was applied to a 0.9 × 215 cm column of Sephacryl S-200 presaturated with BSA and equilibrated with 0.2 M potassium phosphate, pH 6.5, and 1 M NaCl at room temperature. The sample was eluted with the same buffer at a flow rate of 12 mL/h. Fractions (1.6 mL) were collected, and 50-μL aliquots were removed for measurement of radioactivity. The total recovery of radioactivity was 89%.

Scheme II: Major Pathway for Reaction of DTSP-tRNA$_m^{Met}$ with MetRS



DTSP-tRNA$_m^{Met}$ chromatographed in the absence of MetRS. Peak II is not present in unmodified tRNA$_m^{Met}$ but is a higher molecular weight species of free tRNA generated during the coupling of DTSP to the tRNA (data not shown). This species does not participate in the cross-linking reaction to MetRS since the proportion of peak II to the total remains the same after reaction with the enzyme.

DTSP-tRNA$_m^{Met}$ is potentially capable of cross-linking to MetRS by reaction of appropriately oriented lysine ϵ-amino groups with the *N*-hydroxysuccinimide ester moiety or by reaction of cysteine sulfhydryl groups with the disulfide bond of the cross-linker. These two pathways can be distinguished by use of DTSP-tRNA$_m^{Met}$ radioactively labeled with ^{35}S at the two sulfur atoms of the cross-linker. Treatment of the cross-linked complex with DTT leads to cleavage of the disulfide bond and release of free tRNA. Cross-linked lysine residues remain radioactively labeled with one atom of ^{35}S per lysine reacted (Scheme II); however, no ^{35}S remains bound to the protein following DTT cleavage of complexes formed by disulfide exchange. Quantitative measurement of the amount of ^{35}S covalently bound to MetRS before and after treatment with DTT showed that modification of lysine residues accounted for approximately 70% of the total reaction of DTSP-tRNA$_m^{Met}$ with MetRS (Table I).

Reaction of MetRS with DTSP-tRNA$_m^{Met}$ also resulted in loss of the aminoacylation activity of the enzyme. Comparison of the amount of enzyme activity lost with the amount of DTT-stable ^{35}S bound to MetRS showed a 1:1 correspondence

Table I: Cross-Linking of [^{35}S]DTSP-tRNA$_m^{Met}$ to MetRS[a]

	MetRS (μM)	tRNA (μM)[b]	lysine cross-linking[c]		disulfide exchange[d]	
			μM	% of total	μM	% of total
expt 1	1.3	3.1	0.68	69	0.30	31
expt 2	1.3	6.1	1.03	75	0.35	25

[a] Reactions were carried out in 20 mM Hepes, pH 8.0, and 10 mM $MgCl_2$ at 25 °C for 30 min. Samples were quenched by incubation with 50 mM glycine at 25 °C for 30 min. Aliquots were withdrawn and assayed for the amount of enzyme-bound ^{35}S by nitrocellulose filtration. Solutions were adjusted to a final concentration of 20 mM DTT and incubated for 90 min at 25 °C, and additional aliquots were withdrawn for measurement of the amount of DTT-stable ^{35}S bound to MetRS by nitrocellulose filtration. [b] The DTSP-tRNA$_m^{Met}$ concentration is given as the amount of ^{35}S-labeled tRNA added to the reaction, without correction for any hydrolysis of the *N*-hydroxysuccinimide ester group during preparation of the modified tRNA. Such hydrolysis was normally less than 20% of the total labeled tRNA. [c] The amount of lysine cross-linking was calculated from the amount of DTT-stable ^{35}S. [d] The amount of disulfide exchange was calculated from the total cpm of enzyme-bound ^{35}S before DTT minus twice the DTT-stable cpm.

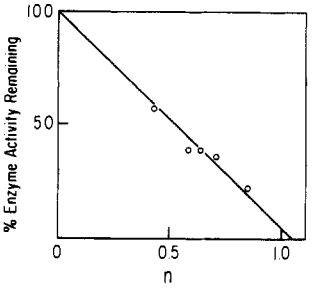


FIGURE 2: Stoichiometry of MetRS inactivation and lysine cross-linking. MetRS and [^{35}S]DTSP-tRNA$_m^{Met}$ were incubated in 20 mM Hepes, pH 8.0, and 10 mM $MgCl_2$ at 25 °C for 20 min. Glycine was added to a final concentration of 50 mM and the incubation continued for 30 min. Aliquots were withdrawn for assay of residual enzyme activity as described under Materials and Methods. The remainder of the reaction mixture was adjusted to 20 mM DTT and incubated at 25 °C for 90 min. Aliquots were removed for determination of the amount of DTT-stable ^{35}S bound to MetRS by nitrocellulose filtration. n = the amount of ^{35}S-labeled MetRS following DTT treatment (e.g., the amount of lysine-cross-linked MetRS) divided by the amount of input MetRS. The data are taken from five separate cross-linking experiments.

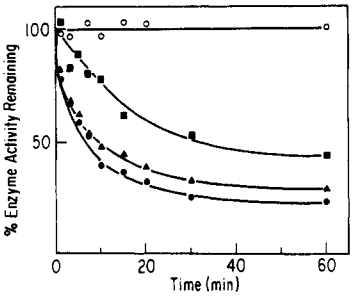


FIGURE 3: Effect of cognate and noncognate tRNAs on the rate of inactivation of MetRS by DTSP-tRNA$_m^{Met}$. Reactions were carried out with 1.1 μM MetRS and 6.6 μM DTSP-tRNA$_m^{Met}$ in 20 mM Hepes, pH 7.4 and 10 mM $MgCl_2$ at 25 °C. Aliquots were removed at various times for measurement of residual enzyme activity as described under Materials and Methods. (●) No additions; (▲), plus 40 μM *E. coli* tRNA^{Phe}; (■) plus 40 μM unmodified *E. coli* tRNA$_m^{Met}$; (○) control minus DTSP-tRNA$_m^{Met}$ plus 10 μM unmodified *E. coli* tRNA^{fMet}.

between enzyme inactivation and lysine cross-linking in five separate experiments (Figure 2). Release of the covalently bound tRNA by DTT treatment resulted in recovery of most of the enzyme activity (70–75%), indicating that inactivation is largely due to the presence of the bound tRNA rather than

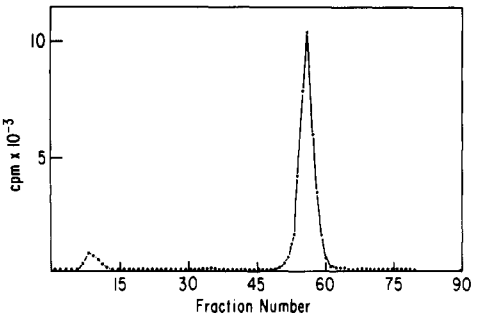
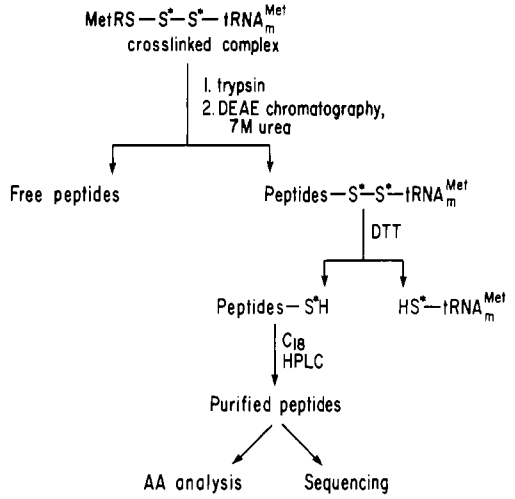


FIGURE 4: Separation of tRNA-bound peptides from free peptides by ion-exchange HPLC. The fraction containing unreacted tRNA and tRNA-bound peptides was dissolved in 7 M urea, 0.1 M NaCl, and 0.05 M ammonium acetate, pH 6.5, and separated from free peptides by chromatography on a column of Bio-Gel TSK-DEAE 5PW under denaturing conditions as described under Materials and Methods. Fractions (0.5 mL) were collected and aliquots (20 μL) withdrawn for measurement of radioactivity.

Scheme III: Isolation of MetRS Peptides Cross-Linked to DTSP-tRNA$_m^{Met}$



to modification of essential amino acid residues.

The specificity of the cross-linking reaction was examined by determining the effect of cognate and noncognate tRNAs on the rate of enzyme inactivation. Reactions were carried out at pH 7.4 in order to reduce the rate of cross-linking, allowing measurement of early time points. Figure 3 shows the rate of inactivation of 1.1 μM MetRS in the presence of 6.6 μM DTSP-tRNA$_m^{Met}$ in 20 mM Hepes, pH 7.4, and 10 mM $MgCl_2$ at 25 °C. Addition of excess unmodified *E. coli* tRNA$_m^{Met}$ (40 μM) to the cross-linking reaction mixture reduced the initial rate of enzyme inactivation approximately 8-fold. In contrast, addition of excess *E. coli* tRNA^{Phe} (40 μM) had no effect on the initial rate of inactivation but partially decreased its final extent (Figure 3).

Purification and Sequencing of a Peptide Cross-Linked to [^{35}S]DTSP-tRNA$_m^{Met}$. The peptides cross-linked to [^{35}S]-DTSP-tRNA$_m^{Met}$ were purified by the procedure outlined in Scheme III. The cross-linked tRNA–enzyme complex was digested with 3% trypsin (w/w) for 7 h at 25 °C. Unreacted tRNA and tRNA-bound peptides were precipitated with ethanol, and the supernatant containing free soluble peptides was removed. The tRNA pellet was dissolved in 7 M urea, 0.1 M NaCl, and 50 mM ammonium acetate, pH 6.5, and separated from the remaining free peptides by chromatography on a Bio-Gel TSK-DEAE 5PW ion-exchange HPLC column under denaturing conditions (Figure 4). Two radioactive peaks eluted from the column. The first peak contained free peptides ^{35}S-labeled by disulfide exchange, plus an excess of free unlabeled peptides. The second peak eluted at the position of unmodified tRNA$_m^{Met}$ and contained free unreacted tRNA and tRNA-bound peptides. The fractions containing tRNA were pooled, dialyzed, and concentrated. The cross-linked peptides were released from the tRNA by cleavage of the disulfide bond following incubation of the sample with 50 mM DTT at 37 °C for 90 min. The free tRNA was precipitated with ethanol, and the supernatant containing the released peptides was concentrated to a small volume and taken up in 6 M GdmCl. The sample was injected onto a reverse-phase HPLC column and eluted with a gradient of acetonitrile (Figure 5). The absorbance at 210 and 280 nm was monitored, and aliquots were taken for measurement of radioactivity. The radioactive peaks eluting from the column prior to fraction 60 did not contain peptide material but were breakdown products derived from the cross-linker side chain released on DTT treatment of the excess unreacted tRNA. Two major peaks of radioactivity eluted in the region of the gradient expected to contain peptides. The absorbance profile showed a prominent peak at 210 nm, which coincided with the radioactive peak at fraction 121. This material was subjected to amino acid composition analysis by a method based on precolumn derivatization with phenyl isothiocyanate (Stone & Williams, 1986). The amino acid analysis (Table II) corresponded to that expected from the tryptic peptide containing a cross-linked lysine at position 596 in the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). The peptide from fraction 121 was also subjected to automatic degradation on an Applied Biosystems gas-phase sequencer (Stone & Williams, 1986). The results of ten cycles of degradation (Table III) confirmed the sequence predicted by the amino acid analysis.

Amino acid composition analysis of the radioactive peak centered at fraction 63 showed only trace amounts of amino acids, indicating that most of the radioactivity in this peak was derived from nonpeptide material. No attempt was made to sequence the minor peaks centered at fractions 84 and 92 due to the low yields. The absorbance profile at 210 nm (Figure 5) suggests that fraction 92 may contain a single peptide. The yield of this peptide is only 25% of the amount of the peptide containing Lys-596.

Table II: Amino Acid Composition Analysis of the Major Cross-Linked Peptide[a]

amino acid	mol/mol of peptide[b]	amino acid	mol/mol of peptide[b]
Asp	1.7 (2)	Met	nd
Glu	3.1 (3)	Cys	nd
Ser	1.6 (1)	Ile	1.0 (1)
Gly	4.1 (1)	Leu	2.5 (3)
His	– (0)	Phe	0.9 (1)
Arg	1.0 (1)	Lys	0.8 (1)
Thr	– (0)	pmol of peptide based on	
Ala	2.1 (2)	radioactivity	120
Pro	– (0)	amino acid analysis	109
Tyr	– (0)		
Val	1.7 (2)		

[a] Analysis of peptide fraction 121 from Figure 5. [b] Values are given as moles of amino acid per mole of peptide. The integer values shown in parentheses represent the theoretical composition for the lysine-containing tryptic peptide Val-Ala-Leu-Ile-Glu-Asn-Ala-Glu-Phe-Val-Glu-Gly-Ser-Asp-Lys$_{596}$*-Leu-Leu-Arg derived from the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). The asterisk indicates the site of the cross-linked lysine. The amide bond of the modified lysine is labile under the conditions used for acid hydrolysis of the peptide, regenerating one lysine residue per mole of peptide. Dashes indicates the value was less than 0.2 mol/mol of peptide. nd indicates not determined.

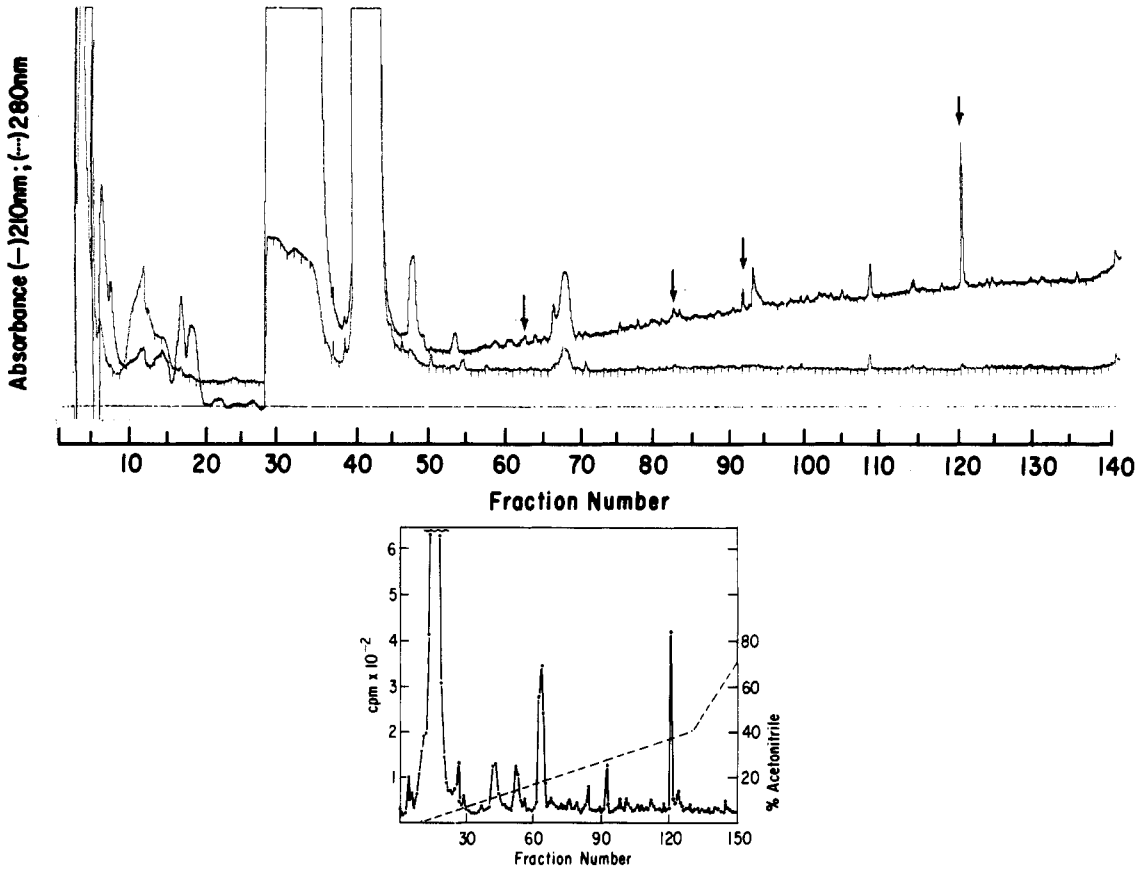


FIGURE 5: Reverse-phase chromatography of MetRS peptides cross-linked to [^{35}S]DTSP-tRNA$_m^{Met}$. The sample in 1.5 mL of 6 M GdmCl was injected onto a reverse-phase HPLC column equilibrated with 0.1% TFA in water (v/v), and the peptides were eluted with a gradient of CH_3CN as described under Materials and Methods. (Top) The absorbance at 210 (upper) and 280 (lower) nm was continuously monitored on a Gilson 116 detector with a full scale of 0.2 OD. Arrows on the absorbance profile indicate the elution positions of the two major and two minor radioactive peaks of fractions 63, 84, 92, and 121. (Bottom) Fractions (0.65 mL) were collected and aliquots (15 μL) withdrawn for measurement of radioactivity.

Table III: Automated Degradation of the Major Cross-Linked Peptide[a]

cycle	residue[b]	amino acid identified	amount (pmol)	yield (%)
1	Val-582	Val	412	100
2	Ala-583	Ala	350	85
3	Leu-584	Leu	272	66
4	Ile-585	Ile	254	61
5	Glu-586	Glu	213	51
6	Asn-587	Asn, Asp	180 (55)	57
7	Ala-588	Ala	229	55
8	Glu-589	Glu	154	37
9	Phe-590	Phe	169	41
10	Val-591	Val	185	45

[a] Peptide fraction 121 (450 pmol) from Figure 5 was subjected to automated degradation on an Applied Biosystems gas-phase sequencer (Stone & Williams, 1986). The amount of peptide was determined from the radioactivity of the sample. Phenylthiodantoin amino acids were determined as described by Merrill et al. (1984). [b] Residue number corresponding to the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984).

Discussion

In this laboratory, new methods for the coupling of cross-linkers to a variety of sites in tRNAs are being developed in order to obtain direct experimental data concerning the relative orientation of peptide and nucleotide sequences at the tRNA binding sites of specific proteins. We have previously described procedures for coupling a variety of affinity-labeling reagents to single-stranded cytidine residues in nucleic acids (Sarkar & Schulman, 1978; Schulman et al., 1981a,b). The cleavable, lysine-reactive cross-linker DTSP proved to be particularly useful for obtaining high-yield cross-linking reactions. A derivative of *E. coli* tRNA^{fMet} carrying this reactive side chain at four different structural regions was found to undergo stoichiometric cross-linking to *E. coli* MetRS by reaction with a limited number of lysine residues in the protein (Schulman et al., 1981a; Valenzuela et al., 1984). Isolation and sequencing of the cross-linked peptides showed that the tRNA was coupled to lysine residues 402, 439, 465, and 640 in the primary sequence of MetRS (Valenzuela & Schulman, 1986).

In this work, we have attached the same lysine-reactive cross-linker to the minor base acp$^3U_{47}$ in the variable loop of *E. coli* tRNA$_m^{Met}$. Coupling of DTSP to the tRNA did not significantly alter its interaction with MetRS, as judged by the ability of an ester-hydrolyzed derivative of DTSP-tRNA$_m^{Met}$ to be aminoacylated with kinetic parameters similar to those of the unmodified tRNA. The extent of reaction of the affinity-labeling tRNA derivative with lysine residues in the protein was followed with [^{35}S]DTSP-tRNA$_m^{Met}$ radioactively labeled in the disulfide bond of the cross-linker. Each covalent linkage through the reactive *N*-hydroxysuccinimide ester group of the tRNA resulted in one DTT-stable ^{35}S-labeled lysine residue in the enzyme. Cross-linking was accompanied by a loss of aminoacylation activity of MetRS. One mole of enzyme was inactivated per mole of lysine-cross-linked tRNA, in keeping with the known anticooperative tRNA binding properties of the native dimeric synthetase. The specificity of the reaction was also indicated by the inhibition of cross-linking on addition of excess unmodified tRNA$_m^{Met}$, but not of a noncognate tRNA.

The major peptide cross-linked to the acp$^3U_{47}$ base was

identified by amino acid analysis and sequencing of the first ten amino acids from the N-terminus. The octadecapeptide corresponds to a tryptic peptide containing a cross-linked lysine residue at position 596 in the primary structure of MetRS. Native MetRS contains two identical subunits of 676 amino acids (Barker et al., 1982; Dardel et al., 1984). Eighty lysine residues (40/subunit) are distributed throughout the primary structure. The flexible side chain on the cross-linker can potentially reach a distance of 14 Å from the acp^{3}U base when fully extended. The small number of lysine residues cross-linked to DTSP-tRNA$_m^{Met}$, with one major site of reaction at Lys-596, further indicates the high degree of specificity of the coupling reaction and places the variable loop of the enzyme-bound tRNA close to the carboxy end of the protein.

It is known that amino acid residues near the carboxy terminus of MetRS are not required for binding or aminoacylation of tRNA^{Met} substrates. Limited digestion of native dimeric MetRS with trypsin produces a monomeric fragment of the enzyme missing approximately 130 amino acids from the carboxy terminus (Waller et al., 1971). This monomeric fragment aminoacylates tRNA^{Met} substrates with kinetic parameters similar to those observed with the native enzyme (Blanquet et al., 1979; Lawrence et al., 1973).

Previous studies on the structural requirements in the variable loop region of methionine tRNAs for aminoacylation by MetRS have shown that chemical modification of U_{47} in tRNA^{fMet} (the base equivalent to acp$^3U_{47}$ in tRNA$_m^{Met}$) has no effect on the biological activity of the tRNA (Schulman & Pelka, 1977a,b; Schulman, 1970). Two different naturally occurring isomers of tRNA^{fMet} are known which contain a single base change at position 46 ($A_{46} \leftrightarrow m^7G_{46}$) (Dube et al., 1968). These two isomers differ in tertiary structure in the central core region of tRNA^{fMet} (Daniel & Cohn, 1976; Delaney et al., 1974; Crothers et al., 1974) and show differences of 1.3–4-fold in K_m and 0–2-fold in V_{max} for aminoacylation, depending on assay conditions (Daniel & Cohn, 1976; Schulman & Pelka, 1977b). Photooxidation of G_{45} in the variable loop of tRNA^{fMet} destroys the purine base and inactivates the tRNA (Schulman, 1971). This base is also believed to be involved in tertiary structure interactions, by analogy with the known crystal structure of yeast tRNA^{Phe} (Quigley & Rich, 1976). Cross-linking of the variable loop region of tRNA$_m^{Met}$ to Lys-596 in a nonessential region of MetRS strongly suggests that no important contacts are made between the protein and this region of the tRNA. Alterations in aminoacylation activity resulting from modification of bases in the variable loop are more likely to reflect conformational changes in the hinge region of the tRNA structure, which alter the position of the 3′ terminus at the active site.

To date, we have identified five peptides in the primary sequence of native MetRS that are covalently coupled to tRNA^{Met} substrates carrying lysine-reactive cross-linkers attached to four different regions of the tRNA structure (Valenzuela & Schulman, 1986; O. Leon & L. H. Schulman, this work and unpublished data). All of the reactive lysine residues are found in the carboxy-terminal half of the protein, indicating that this region of MetRS contains the tRNA binding site. The monomeric form of the enzyme has been crystallized and its structure determined at 2.5-Å resolution (Zelwer et al., 1982). Refinement of the structure at 1.8-Å resolution is near completion, and three of the cross-linked lysine residues have recently been located in the three-dimensional structure (S. Brunie, J. L. Risler, and C. Zelwer, unpublished results). In addition, Hountondji et al. (1985) have cross-linked periodate-oxidized tRNA^{fMet} to the monomeric fragment of MetRS and identified three cross-linked peptides near the active site of the enzyme. The peptides found in highest yield were coupled to the 3′ terminus of the tRNA through Lys-335 and Lys-61.

Cross-links to amino acid residues present in the monomeric fragment of MetRS are currently being used to construct a model of the tRNA–synthetase complex. It has not yet been possible to obtain crystals of native MetRS that are suitable for high-resolution X-ray analysis or cocrystals of tRNA bound to either form of the enzyme. In solution, only one molecule of tRNA^{Met} is bound per molecule of dimeric synthetase, leaving one of the subunits unoccupied (Blanquet et al., 1973). The cross-links obtained to sequences near the carboxy terminus of the native enzyme may assist in determining the orientation of the two subunits when the dimeric synthetase is complexed to its cognate tRNA.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Ken Williams and Kathy Stone at the Yale University Protein Sequencing Facility in the analysis of the cross-linked peptides. We thank Heike Pelka for helpful discussions and for assistance in the preparation of MetRS. We also thank Rita Romita for expert typing.

Registry No. DTSP, 57757-57-0; acp^{3}U, 52745-94-5; MetRS, 9033-22-1; L-Lys, 56-87-1.

REFERENCES

Barker, D. G., Ebel, J.-P., Jakes, R., & Bruton, C. J. (1982) *Eur. J. Biochem. 127*, 449–457.

Blanquet, S., Iwatsubo, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36*, 213–226.

Blanquet, S., Dessen, P., & Fayat, G. (1979) in *Transfer RNA: Structure, Properties and Recognition* (Schimmel, P. R., Soll, D., & Abelson, J. N., Eds.) pp 281–294, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Budker, V. G., Knorre, D. G., Kravchenko, V. V., Lavrik, O. I., Nevinsky, G. A., & Teplova, N. M. (1974) *FEBS Lett. 49*, 159–162.

Crothers, D. M., Cole, P. E., Hilbers, C. W., & Shulman, R. G. (1974) *J. Mol. Biol. 87*, 63–88.

Daniel, W. E., Jr., & Cohn, M. (1976) *Biochemistry 15*, 3917–3924.

Dardel, F., Fayat, G., & Blanquet, S. (1984) *J. Bacteriol. 160*, 1115–1122.

Delaney, P., Bierbaum, J., & Ofengand, J. (1974) *Arch. Biochem. Biophys. 161*, 260–267.

Dube, S. K., Marcker, K. A., Clark, B. F. C., & Cory, S. (1968) *Nature (London) 218*, 232–233.

Duffy, L. K., Gerber, L., Johnson, A. E., & Miller, D. L. (1981) *Biochemistry 20*, 4663–4666.

Friedman, S. (1973) *Nature (London), New Biol. 244*, 18–20.

Friedman, S., Li, H. J., Nakanishi, K., & VanLear, G. (1974) *Biochemistry 13*, 2932–2937.

Gorshkova, I. I., Knorre, D. G., Lavrik, O. I., & Nevinsky, G. A. (1976) *Nucleic Acids Res. 3*, 1577–1589.

Hountondji, C., Blanquet, S., & Lederer, F. (1985) *Biochemistry 24*, 1175–1180.

Hountondji, C., Lederer, F., Dessen, P., & Blanquet, S. (1986) *Biochemistry 25*, 16–21.

Kao, T.-H., Miller, D. L., Abo, M., & Ofengand, J. (1983) *J. Mol. Biol. 166*, 383–405.

Lawrence, F., Blanquet, S., Poiret, M., Roberto-Gero, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36*, 234–243.

Lomant, A. J., & Fairbanks, G. (1976) *J. Mol. Biol. 104*, 243–261.

Merrill, B. M., Williams, K. R., Chase, J. W., & Konigsberg, W. H. (1984) *J. Biol. Chem. 259*, 10850–10856.
Ohashi, Z., Maeda, M., McCloskey, J. A., & Nishimura, S. (1974) *Biochemistry 13*, 2620–2625.
Quigley, G. J., & Rich, A. (1976) *Science (Washington, D.C.) 194*, 796–806.
Renaud, M., Fasiolo, F., Baltzinger, M., Boulanger, Y., & Remy, P. (1982) *Eur. J. Biochem. 123*, 267–274.
Sarkar, A. K., & Schulman, L. H. (1978) *Methods Enzymol. 59*, 156–166.
Schulman, L. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 507–514.
Schulman, L. H., & Pelka, H. (1977a) *J. Biol. Chem. 252*, 814–819.
Schulman, L. H., & Pelka, H. (1977b) *Biochemistry 16*, 4256–4265.
Schulman, L. H., & Pelka, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6755–6759.
Schulman, L. H., Valenzuela, D., & Pelka, H. (1981a) *Biochemistry 20*, 6018–6023.
Schulman, L. H., Pelka, H., & Reines, S. A. (1981b) *Nucleic Acids Res. 9*, 1203–1217.
Stone, K. L., & Williams, K. R. (1986) *J. Chromatogr. 359*, 203–212.
Valenzuela, D., & Schulman, L. H. (1986) *Biochemistry 25*, 4555–4561.
Valenzuela, D., Leon, O., & Schulman, L. H. (1984) *Biochem. Biophys. Res. Commun. 119*, 677–684.
VanNoort, J. M., Kraal, B., Bosch, L., LaCour, T. F. M., Nyborg, J., & Clark, B. F. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3969–3972.
Waller, J. P., Risler, J. L., Monteilhet, C., & Zelwer, C. (1971) *FEBS Lett. 16*, 186–188.
Zelwer, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81.

Glucosamine Synthetase from *Escherichia coli*: Purification, Properties, and Glutamine-Utilizing Site Location

Bernard Badet,*,‡ Patricia Vermoote,‡ Pierre-Yves Haumont,§ Florence Lederer,§ and Francois Le Goffic‡
Laboratoire de Bioorganique et Biotechnologies, ENSCP, 75231 Paris Cedex 05, France, and Hopital Necker, INSERM U.25, CNRS LA 122, 75730 Paris Cedex 15, France

Received July 28, 1986; Revised Manuscript Received November 13, 1986

ABSTRACT: L-Glutamine:D-fructose-6-phosphate amidotransferase (glucosamine synthetase) has been purified to homogeneity from *Escherichia coli*. A subunit molecular weight of 70 800 was estimated by gel electrophoresis in sodium dodecyl sulfate. Pure glucosamine synthetase did not exhibit detectable NH_3-dependent activity and did not catalyze the reverse reaction, as reported for more impure preparations [Gosh, S., Blumenthal, H. J., Davidson, E., & Roseman, S. (1960) *J. Biol. Chem. 235*, 1265]. The enzyme has a K_m of 2 mM for fructose 6-phosphate, a K_m of 0.4 mM for glutamine, and a turnover number of 1140 min^{-1}. The amino-terminal sequence confirmed the identification of residues 2–26 of the translated *E. coli glmS* sequence [Walker, J. E., Gay, J., Saraste, M., & Eberle, N. (1984) *Biochem. J. 224*, 799]. Methionine-1 is therefore removed by processing in vivo, leaving cysteine as the NH_2-terminal residue. The enzyme was inactivated by the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) and by iodoacetamide. Glucosamine synthetase exhibited half-of-the-sites reactivity when incubated with DON in the absence of fructose 6-phosphate. In its presence, inactivation with [6-^{14}C]DON was accompanied by incorporation of 1 equiv of inhibitor per enzyme subunit. From this behavior, a dimeric structure was tentatively assigned to the native enzyme. The site of reaction with DON was the NH_2-terminal cysteine residue as shown by Edman degradation.

L-Glutamine:D-fructose-6-phosphate amidotransferase (EC 2.6.1.16) catalyzes the first reaction in hexosamine biosynthesis (eq 1). It belongs to the group of glutamine amidotransferases

L-glutamine + D-fructose 6-phosphate → D-glucosamine 6-phosphate + L-glutamate (1)

which utilize the amide of glutamine in the biosynthesis of amino acids, nucleotides, and coenzymes (Buchanan, 1973; Prusiner & Stadman, 1973). This enzyme is unique among this group in the fact it is the only one transferring the amide nitrogen to a keto group without the participation of a cofactor. The product, glucosamine 6-phosphate ($GlcNH_2$-6-P),[1] undergoes sequential transformations leading to the formation of UDP-*N*-acetylglucosamine, the major intermediate in the biosynthesis of all amino sugar containing macromolecules both in prokaryotic and in eukaryotic cells. Therefore, the

* Address correspondence to this author.
‡ Laboratoire de Bioorganique et Biotechnologies, ENSCP.
§ Hopital Necker, INSERM U.25.

[1] Abbreviations: APAD, acetylpyridine adenine dinucleotide; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyanate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiohydantoin; DON, 6-diazo-5-oxo-L-norleucine; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; Fru-6-P, fructose 6-phosphate; $GlcNH_2$-6-P, glucosamine 6-phosphate; Gln, L-glutamine; IAAm, iodoacetamide; PMSF, phenylmethanesulfonyl fluoride; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis; Cam, carbamoylmethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; kDa, kilodalton(s).

0006-2960/87/0426-1940$01.50/0